

1 **IRF5 promotes influenza-induced inflammatory responses in human iPSC-derived**  
2 **myeloid cells and murine models**

4 **Running title: IRF5 promotes influenza-induced inflammatory responses**

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25

26 **Abstract**

27 Recognition of Influenza A virus (IAV) by the innate immune system triggers pathways that  
28 restrict viral replication, activates innate immune cells, and regulates adaptive immunity.  
29 However, excessive innate immune activation can exaggerate disease. The pathways  
30 promoting excessive activation are incompletely understood, with limited experimental  
31 models to investigate mechanisms driving influenza-induced inflammation in humans.  
32 Interferon regulatory factor (IRF5) is a transcription factor that plays important roles in  
33 induction of cytokines after viral sensing. In an *in vivo* model of IAV infection, IRF5  
34 deficiency reduced IAV-driven immune pathology and associated inflammatory cytokine  
35 production, specifically reducing cytokine-producing myeloid cell populations in *Irf5*<sup>-/-</sup> mice,  
36 but not impacting type 1 IFN production or virus replication. Using cytometry by time-of-  
37 flight (CyTOF), we identified that human lung IRF5 expression was highest in cells of the  
38 myeloid lineage. To investigate the role of IRF5 in mediating human inflammatory responses  
39 by myeloid cells to IAV, we employed human induced pluripotent stem cells (hiPSCs) with  
40 biallelic mutations in *IRF5*, demonstrating for the first time iPS-derived dendritic cells (iPS-  
41 DCs) with biallelic mutations can be used to investigate regulation of human virus-induced  
42 immune responses. Using this technology, we reveal that IRF5 deficiency in human DCs, or  
43 macrophages, corresponded with reduced virus-induced inflammatory cytokine production,  
44 with IRF5 acting downstream of TLR7 and, possibly, RIG-I after viral sensing. Thus, IRF5  
45 acts as a regulator of myeloid cell inflammatory cytokine production during IAV infection in  
46 mice and humans, and drives immune-mediated viral pathogenesis independently of type 1  
47 IFN and virus replication.

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## 51 **Importance**

52 The inflammatory response to Influenza A virus (IAV) participates in infection control but  
53 contributes to disease severity. After viral detection intracellular pathways are  
54 activated, initiating cytokine production, but these pathways are incompletely  
55 understood. We show that interferon regulatory factor 5 (IRF5) mediates IAV-induced  
56 inflammation and, in mice, drives pathology. This was independent of antiviral type 1 IFN  
57 and virus replication, implying that IRF5 could be specifically targeted to treat influenza-  
58 induced inflammation. We show for the first time that human iPSC technology can be  
59 exploited in genetic studies of virus-induced immune responses. Using this technology, we  
60 deleted IRF5 in human myeloid cells. These IRF5-deficient cells exhibited impaired  
61 influenza-induced cytokine production and revealed that IRF5 acts downstream of Toll-like  
62 receptor 7 and possibly retinoic acid-inducible gene-I. Our data demonstrate the importance  
63 of IRF5 in influenza-induced inflammation, suggesting genetic variation in the IRF5 gene  
64 may influence host susceptibility to viral diseases.

65

## 66 **Introduction**

67 During infection with Influenza A virus (IAV), the host immune system must calibrate  
68 immune responses to control viral infection, whilst minimizing damage to host tissues.  
69 Disease manifestations are often associated with host inflammatory response to the virus (1,  
70 2), and clinical outcome varies widely between individuals (3). The inflammatory response is  
71 initiated when pattern recognition receptors (PRRs) on innate immune cells recognize IAV  
72 pathogen-associated molecular patterns (PAMPs), which trigger signaling cascades resulting  
73 in the expression of specific inflammatory cytokines and chemokines (4, 5). Cytokines play  
74 various roles, such as directly inhibiting viral replication and activating the cytolytic

75 functions of T-cells, whereas chemokines recruit innate immune cells such as macrophages,  
76 neutrophils, NK cells and inflammatory monocytes to the lungs and airways (6).

77

78 Interferon regulatory factor 5 (IRF5) is a member of the IRF family of transcription factors,  
79 whose members have a shared N-terminal DNA binding domain and bind consensus  
80 interferon stimulated response element (ISRE) motifs. As ISREs are enriched in the  
81 regulatory regions of immune genes, IRFs play key roles as master regulators in the innate  
82 immune response (7), and may provide a mechanism for conferring signal specificity to  
83 target gene sets downstream of TLR signaling (8). Whilst IRF3 and IRF7 are necessary for  
84 induction of type I interferon (9, 10), IRF5 has been shown to be key in regulating  
85 inflammatory cytokine responses, generally acting downstream of TLR-MyD88 pathways  
86 (11, 12). Genetic polymorphisms in the IRF5 gene in humans have been linked to various  
87 autoimmune conditions including systemic lupus erythematosus, rheumatoid arthritis,  
88 Sjogren's syndrome, multiple sclerosis and inflammatory bowel disease (13). IRF5 has also  
89 been shown to be important in regulating immune responses to various pathogens in murine  
90 and human cell models (14–17). Additionally, *Ir5*<sup>-/-</sup> mice are resistant to systemic shock  
91 induced by CpG ligands and Lipopolysaccharide (LPS) (12). The extent to which IRF5  
92 contributes to inflammation-induced pathologies, however, is unclear.

93

94 IRF5 is expressed predominantly by myeloid cells such as dendritic cells (DCs) and  
95 macrophages, in addition to B cells (13, 18). Myeloid cells can have protective and immune-  
96 pathogenic roles during IAV infection, producing inflammatory cytokines and initiating  
97 adaptive immune responses (19). Furthermore, inflammatory monocytes and monocyte-  
98 derived DCs have been identified to drive inflammation and lung pathology during IAV  
99 infection (19, 20).

100 Studying inflammatory cytokine responses in human myeloid cells is challenging. Human  
101 DCs are difficult to culture *in vitro* and, although DCs can be induced from blood-derived  
102 monocytes, these cells display morphological and functional differences to human primary  
103 DCs, for example differing in their capacity for T cell stimulation in comparison to CD11c<sup>+</sup>  
104 blood-derived DCs (21). Primary myeloid cells are also difficult to genetically manipulate,  
105 meaning that studies addressing the effect of host genetics on myeloid cell responses can be  
106 challenging. Human induced pluripotent stem cells (hiPSCs) offer a useful system for  
107 studying host-pathogen variation, because these cells are amenable to genetic manipulation,  
108 can be differentiated toward multiple cellular lineages and are self-renewing, allowing for  
109 production of sufficient quantities of cells of the same genetic background. hiPSC-derived  
110 macrophages (iPSDMs) have already been used to successfully model the interactions of  
111 pathogens with host cells (16, 22). However, to date, hiPSC technology has not been used to  
112 perform genetic investigations of virus-induced immune responses. To study the impact of  
113 IRF5 on human myeloid IAV-induced immune responses, we utilized hiPSCs generated from  
114 a healthy donor, or with mutations in *IRF5* generated by CRISPR/Cas9 engineering,  
115 differentiated into dendritic cells and macrophages as a human model system to assess the  
116 role of IRF5 in the regulation of immune responses to IAV. Using these tools in combination  
117 with studies of human lung cells, in addition to *Irf5*<sup>-/-</sup> mice, we show that IRF5 drives IAV-  
118 induced inflammatory cytokine responses in mice and humans without impacting virus  
119 replication and type 1 IFN secretion, and this process mediates viral pathogenesis *in vivo*.

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## 125 **Materials and Methods**

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### 127 **Mice and viral infections**

128 *Irf5*<sup>-/-</sup> mice were bred in-house on a C57BL/6 background and their generation has been  
129 described previously (12). C57BL/6 WT mice were purchased from Charles River or Envigo.  
130 Age- and sex-matched mice between 7 and 12 weeks of age were used in the experiments.  
131 Mice were infected intranasally with 3 x 10<sup>3</sup> PFU A/X-31 influenza in 50µl of sterile PBS.  
132 Mice weight was recorded daily and further monitored for signs of illness.

133

### 134 **Plaque assays**

135 Influenza from lungs of WT and *Irf5*<sup>-/-</sup> mice was quantified on Madin–Darby canine kidney  
136 (MDCK) cell monolayers after a 5-hour incubation at 37°C. Cell layers were then overlaid  
137 with methylcellulose (Sigma-Aldrich) and incubated at 37 °C for a further 48 hours. Media  
138 was then removed, and cell layers were washed, fixed and blocked and further incubated with  
139 anti-Influenza A nucleoprotein (Clone AA5H, Serotec) and then with anti-mouse IgG-HRP  
140 (BioRad). Plaques were developed using AEC peroxidase substrate solution and subsequently  
141 counted via light microscopy.

142

### 143 **Leukocyte isolation, intracellular cytokine staining, and flow cytometry**

144 BAL and lungs were collected from *Irf5*<sup>-/-</sup> and WT mice at days 2, 4 and 7 p.i. Lung digests  
145 were performed by incubation with collagenase solution (RPMI supplemented with 5% FBS,  
146 1mg/ml collagenase D (Roche), 5 mM CaCl<sub>2</sub>, 50 mg/mL DNase I (Sigma-Aldrich)), and  
147 single cell suspensions were generated by passing through 100 µM filters. Cells were stained  
148 with Zombie Aqua™ fixable dye, incubated with anti-mouse CD16/CD32 (both BioLegend),

149 and stained for surface markers with a combination of the following antibodies (all  
150 BioLegend, BD Biosciences or Miltenyi Biotec). For murine myeloid panels cells were  
151 stained for surface markers: anti-mouse CD11b-FITC (M1/70, BioLegend), Ly6C-  
152 PerCP/Cy5.5 (HK1.4, BioLegend), Siglec F-PeVio770 (ES22-10D8, Miltenyi Biotec),  
153 CD64-Pe/Dazzle (X54-5/7.1, BioLegend), CD45R/B220-APC/Cy7 (RA3-6B2, BioLegend),  
154 MHC II-BV711 (M5/114.15.2, BioLegend), CD11c-BV605 (N418, BioLegend) and Siglec  
155 H-Pacific Blue (551, BioLegend). Following surface staining, some cells were fixed and  
156 permeabilized with Fixation/Permeabilization solution (BD Biosciences) and stained with  
157 anti-IL-6-PE (MP5-20F3; BioLegend) and TNF- $\alpha$ -APC (MP6-XT22, BioLegend). All data  
158 was acquired using an Attune NxT flow cytometer (Thermo Fisher Scientific). Electronic  
159 compensation was performed with Ab capture beads stained separately with individual mAbs  
160 used in the experimental panel. Data were analyzed using FlowJo software (TreeStar Inc).  
161 Total numbers of different cell populations were calculated by multiplying the total number  
162 of viable leukocytes (assessed by trypan blue exclusion) by the percentage of positive cells,  
163 as detected by flow cytometry.

164

#### 165 **Mass Cytometry staining for IRF5 expression**

166 Para-tumor lung tissue samples from metastatic cancer or fibrosis patients were extracted and  
167 deemed to show no visible signs of inflammation by a pathologist. PBMC from one donor  
168 were run with each lung sample to control for inter-run variability. 100 mL of heparinized  
169 blood was drawn from a healthy control donor, PBMCs were isolated and aliquots were  
170 frozen until use. Directly conjugated antibodies (CD45-89Y, clone HI30; EpCAM-141Pr,  
171 clone 9C4; CD31-151Eu, clone EPR3094; CD68-159Tb, clone KP1; Siglec 8-164Dy, clone  
172 7C9) were all purchased from Fluidigm. Conjugations for other antibodies (CD11b-142Nd,  
173 clone ICRF44; CD4-145Nd, clone RPA-T4; CD20-147Sm, clone 2H7; CD115-152Sm, clone

174 12-3A3-1810; CD123-155Gd, clone 6H6; CD14-160Gd, clone M5P2; CD56-166Er, clone  
175 NCM/HCD56; CD8-168Er, clone SK1; HLA-DR-169Tm, clone L243; CD3-170Er, clone  
176 UCHT1; CD1c-171Yb, clone L161; CD141-173Yb, clone M80) were performed with the  
177 Maxpar X8 Multi-Metal Labeling Kit (Fluidigm) according to the manufacturer's  
178 instructions.

179

180 Cells re-suspended at  $1 \times 10^7$  cells/mL were stained with 5 mmol/L Cisplatin (Fluidigm;  
181 live/dead) and surface antibody cocktail before permeabilization with Maxpar nuclear antigen  
182 staining buffer and staining with anti-IRF5 (Conjugate, 175Lu; clone, EPR17067). An un-  
183 permeabilized control was treated with cell staining buffer and stained with intracellular  
184 antibodies. Cells were stained with 125 nM Ir-Intercalator (Fluidigm) according to Fluidigm  
185 protocols and fixed with 1.6% formaldehyde. Cells were counted on a BD Accuri C6, and  
186 resuspended at  $2 \times 10^6$  cells/mL in 0.1 $\times$ EQ Four Element Calibration Beads (Fluidigm). Cells  
187 were acquired using a CyTOF Helios cytometer (Fluidigm). Data was processed and  
188 normalized using the CyTOF software v6.7 (Fluidigm). Data was analyzed using FlowJo  
189 (Treestar Inc).

190

### 191 **Mass cytometry Analysis**

192 CyTOF data were analyzed using FlowJo 10.5.2 (TreeStar Inc). After gating on live, intact,  
193 singlet cells, CD45 v EpCAM expression was used to identify epithelial cells (CD45<sup>-</sup>  
194 EpCAM<sup>+</sup>) or immune cells (CD45<sup>+</sup>EpCAM<sup>-</sup>). CD45<sup>+</sup>EpCAM<sup>-</sup> cells were down-sampled to  
195 maximum 200,000 per sample, with stained and control samples were concatenated into one  
196 file. The concatenated file was run through a UMAP analysis of the surface markers CD11b,  
197 CD4, CD20, CD123, CD68, CD14, Siglec 8, CD56, CD8, HLA-DR, CD3, CD1c, CD141,  
198 CD16. Post-UMAP analysis, distinct cell subsets were identified by mapping expression of



specific subset markers back onto the UMAP to define populations of immune cells. Subsets were defined as follows: CD4<sup>+</sup> T cells, CD3<sup>+</sup>CD4<sup>+</sup>CD20<sup>-</sup>; CD8<sup>+</sup> T cells, CD3<sup>+</sup>CD20<sup>-</sup>CD8<sup>+</sup>; B cells, CD3<sup>-</sup>CD20<sup>+</sup>; NK cells, CD3<sup>-</sup>CD20<sup>-</sup>CD56<sup>+</sup>; CD14<sup>+</sup> Monocytes, CD16<sup>-</sup>CD11b<sup>+</sup>CD14<sup>+</sup>HLA-DR<sup>+</sup>; CD16<sup>+</sup> Monocytes, CD14<sup>-</sup>CD11b<sup>+</sup>CD16<sup>+</sup>HLA-DR<sup>+</sup>; Macrophages, CD11b<sup>+</sup>CD68<sup>+</sup>HLA-DR<sup>+</sup>; pDCs, CD123<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>+</sup>; CD141<sup>+</sup> cDCs, CD11b<sup>+</sup>HLA-DR<sup>+</sup>CD1c<sup>-</sup>CD141<sup>+</sup>; CD1c<sup>+</sup> cDCs, CD11b<sup>+</sup>HLA-DR<sup>+</sup>CD1c<sup>+</sup>CD141<sup>-</sup>; Eosinophils, Siglec8<sup>+</sup>CD123<sup>-</sup>; Basophils, Siglec8<sup>+</sup>CD123<sup>+</sup>; Epithelial cells, CD45<sup>-</sup> EpCAM<sup>+</sup>. Individual samples were identified by gating on event length v sample ID, and the median value was determined for IRF5 for each individual sample.

#### Generation of blood-derived human dendritic cells

PBMCs from three independent donors were isolated from leukapheresis products using Lymphoprep density gradient centrifugation and SepMate PBMC isolation tubes (StemCell Technologies), under the Weatherall Institute of Molecular Medicine, University of Oxford Human Tissue Authority License (12433). Human CD14 microbeads were used in combination with LS columns (both Miltenyi Biotec) to positively select CD14<sup>+</sup> blood monocytes. CD14<sup>+</sup> cells were seeded at a density 3-5×10<sup>6</sup> isolated monocytes in 3 mL of RPMI media supplemented with 10% heat inactivated Fetal Bovine Serum (FBS; Sigma-Aldrich), 250 IU/mL IL-4 and 800 IU/mL GM-CSF into a 6-well plate and incubated at 37°C for 2 days. After 2 days 1.5ml of medium was removed from each well, and 1.5 mL of fresh medium supplemented with 500 IU/mL IL-4 and 1600 IU/mL GM-CSF was added. After a further 3-day incubation, cells were harvested at the immature phenotype and assayed, or further matured with LPS at 10 µg/mL for 24 hours.

## 224 **hiPSCs**

225 The healthy control hiPSC line Kolf2 was acquired through the Human Induced Pluripotent  
226 Stem Cells Initiative Consortium (HipSci; [www.hipsci.org](http://www.hipsci.org)), through which it was also  
227 characterized (23). Generation of IRF5<sup>-/-</sup> iPSCs has been previously described (16). Briefly,  
228 Kolf2 iPSCs were dissociated to single cells and nucleofected (Amaxa2b nucleofector,  
229 LONZA) with Cas9 coding plasmid (hCas9, Addgene 41815), sgRNA plasmid (left  
230 CRISPR\_IRF5 CCAAGTGGAAGGCCAACCTGCGC; right CRISPR\_IRF5  
231 GACTTCCGCCTCATCTACGACGG) and donor plasmid, containing 5' and 3' homology  
232 arms for IRF5 targeting and pL1-EF1αPuro-L2 cassette. Post nucleofection, cells were  
233 selected for up to 11 days with 0.25 µg/mL puromycin, after which individual colonies were  
234 picked onto 96-well plates, grown to confluence and then replica plated. To genotype  
235 individual clones from a 96-well replica plates, cells were lysed and used for PCR  
236 amplification with LongAmp Taq DNA Polymerase (NEB). Insertion of the cassette into the  
237 correct locus was confirmed by visualizing on 1% E-gel (Life Tech.) PCR products were  
238 generated by gene specific and cassette specific primers, with single integration of cassette  
239 confirmed by a qPCR copy number assay. Positive clones were then screened for damage to  
240 the non-targeted allele via PCR and Sanger sequencing. To generate our complemented IRF5  
241 iPSC line (IRF5<sup>Comp</sup>) and restore expression of functional IRF5 in the IRF5<sup>-/-</sup> iPSCs, we  
242 generated the AAVS1 EF1α-IRF5-PGK-puro targeting vector by Gibson assembly. The  
243 Gibson assembly product was transformed into OneShot TOP10 chemically competent *E.*  
244 *coli* (Thermo Fisher Scientific) and positive colonies were picked. Isolated plasmids from the  
245 positive colonies were taken to confirm the presence and sequence of EF1α-IRF5 in the  
246 targeting vector by restriction digests, PCR and sequencing. Subsequently, the targeting  
247 vector was transformed into competent *E. coli* to isolate endotoxin-free plasmids to transform  
248 into the IRF5<sup>-/-</sup> iPSCs. We transfected the mutant human hiPSCs with TALEN-L

(CCCCTCCACCCCACAGT), TALEN-R (AGGATTGGTGACAGAAA) and targeting vector via nucleofection (Amaxa Biosystems). The resultant targeted cells were selected on puromycin for 7 days and the surviving colonies were picked and expanded. The positive clones were confirmed by RT-qPCR for *IRF5* expression and flow cytometry for protein expression. Prior to differentiation, iPSCs were grown feeder-free using the Essential 8 Flex Medium kit (Thermo Fisher Scientific) on Vitronectin (VTN-N, Thermo Fisher Scientific) coated plates as per manufacturer's instructions to 70-80% confluency. iPSCs were harvested for differentiation using Versene solution (Thermo Fisher Scientific).

257

#### 258 **Differentiation of iPSCs to dendritic cells and macrophages**

259 To differentiate iPSCs to dendritic cells slight modifications were made to a previously  
260 published protocol (24). Briefly, upon reaching confluency iPSCs were harvested and plated  
261 into Essential 8 Flex medium supplemented with 50 ng/mL BMP-4 (Bio-Techne), 20 ng/mL  
262 SCF (Bio-Techne), 50 ng/mL VEGF (Peprotech EC Ltd.), and 50ng/mL GM-CSF (Peprotech  
263 EC Ltd.) in ultra-low attachment plates (Corning). Media was changed to X-VIVO-15  
264 (Lonza), with sequential removal of BMP-4 by day 5, VEGF by approximately day 14 and  
265 SCF by approximately day 19. In addition, IL-4 (Peprotech EC Ltd.) was added sequentially  
266 in increasing concentrations, starting from approximately day 12 at 25 ng/mL and increasing  
267 to 100 ng/mL by approximately day 20. By day 20, floating immature DCs were harvested  
268 from ULA plates, filtered through 70µM filters (Corning), counted and seeded at  $1 \times 10^6$  per  
269 well of 6 well CellBind plates (Corning) in X-VIVO-15 media supplemented with 100 ng/mL  
270 IL-4 and 50 ng/mL GM-CSF. iPS-DCs were used for assays at the immature phase between  
271 4-5 days post seeding in CellBind plates. In addition, iPS-DCs could be matured for a further  
272 48 hours 5 days post plating, by addition of 50 ng/ml of GM-CSF, 100 ng/ml IL-4, 20 ng/ml  
273 IFN $\gamma$  (Bio-Techne), 50 ng/ml TNF $\alpha$  (Bio-Techne), 10 ng/ml IL-1 $\beta$  (Bio-Techne) and 1 µg/ml

274 PGE<sub>2</sub> (Sigma-Aldrich), to induce further expression of CD141<sup>+</sup> DC lineage markers. For  
275 assays, floating iPS-DCs were harvested from differentiation plates, washed with PBS,  
276 counted and seeded in X-VIVO-15 media without cytokines at an assay dependent  
277 concentration. To differentiate iPSCs to macrophages, the approach of Hale et al (22) and van  
278 Wilgenburg et al (25) was modified to allow for feeder-free differentiation. Briefly, upon  
279 reaching confluency, human iPSCs were collected and transferred into Essential 8 Flex  
280 medium supplemented with 50 ng/mL BMP-4 (Bio-Techne), 20 ng/mL SCF (Bio-Techne)  
281 and 50 ng/mL VEGF (Peprotech EC Ltd.) in ultra-low attachment plates (Corning) for 4 days  
282 to generate Embryoid Bodies (EBs). On day 5, EBs were used for generation of myeloid  
283 precursor cells by plating into 6-well tissue culture treated plates (Corning) coated for two  
284 hours at room temperature with 0.1% gelatin, in X-VIVO-15 media supplemented with  
285 25 ng/mL IL-3 (Bio-Techne) and 50 ng/mL M-CSF (Bio-Techne). After several weeks,  
286 floating myeloid precursors were harvested and terminally differentiated into matured  
287 macrophages (iPSDMs) in the presence of higher concentrations of M-CSF (100 ng/mL) for 7  
288 days. For experiments, macrophages were detached using Lidocaine solution (4 mg/mL  
289 lidocaine-HCl with 10 mM EDTA in PBS), and seeded at  $2 \times 10^5$  cells per well (24-well  
290 plate) or  $1 \times 10^6$  cells per well (six-well plate).

291

#### 292 **Preparation of RNA and RT-qPCR**

293 iPS-DCs were harvested from plates and RNA was prepared using the RNeasy minikit  
294 (Qiagen). RNA was reverse transcribed with the QuantiTect reverse transcription (RT) kit  
295 (Qiagen) according to the manufacturer's protocol. All RT-qPCR experiments were  
296 performed with TaqMan gene expression assays and TaqMan gene expression master mix  
297 (Applied Biosystems) on the Applied Biosystems StepOne real-time PCR system. RT-qPCR

298 data were analyzed via the comparative CT method with glyceraldehyde 3-phosphate  
299 dehydrogenase (GAPDH) as an endogenous control.

300

### 301 **Flow cytometric analysis of iPS-DCs**

302 For analysis of surface markers on iPS-DCs, cells were stained with Zombie Aqua™ fixable  
303 dye (BioLegend), Fc receptors were blocked using Human TruStain FcX (BioLegend) and  
304 cells were then subsequently stained for surface markers with a combination of the following  
305 antibodies: anti-human HLA-DR-AlexaFluor488 (AF488) (L243, BioLegend) or CD14-FITC  
306 (M5E2, BioLegend), CD83-PerCP/Cy5.5 (HB15e, BioLegend) or CD1c-PerCP/Cy5.5 (L161,  
307 BioLegend), CD141-PE/Cy7 (M80, BioLegend) or DC-SIGN-PE/Cy7 (9E9A8, BioLegend),  
308 or XCR1-PE (FAB8571, Bio-Techne), CD11c-APC/Cy7 (Bu15, BioLegend), CLEC9A-APC  
309 (8F9, BioLegend), CD86-BV711 (IT2.2, BioLegend), CD303-BV785 (201A, BioLegend) or  
310 HLA-DR-BV785 (L243, BioLegend), or HLA-A,B,C-Pacific Blue (W6/32, BioLegend). For  
311 detection of IRF5 or IAV nucleoprotein, cells were stained with Zombie Aqua™ fixable dye,  
312 fixed with 4% Paraformaldehyde and permeabilized with 0.5% Triton X, followed by  
313 incubation with Human TruStain FcX and staining with IRF5-AF647 (EPR6094, Abcam) or  
314 Anti-Influenza A Virus Nucleoprotein antibody (431, Abcam) in 0.1% Triton X solution. All  
315 data was acquired using a BD LSRFortessa flow cytometer (BD Biosciences). Electronic  
316 compensation was performed with Ab capture beads stained separately with individual mAbs  
317 used in the experimental panel. Data were analyzed using FlowJo software (TreeStar Inc).

318

### 319 **Infection of iPS-DCs and iPSDMs with IAV**

320 iPS-DCs, iPSDMs or human moDCs were infected with A/X-31 influenza at an MOI of 1 by  
321 addition of virus to culture supernatant and centrifugation at 630 g for 20 minutes at room  
322 temperature, after which media was replaced with fresh culture medium.

323 **Immunostaining for confocal microscopy**

324 iPS-DCs were harvested from plates and spun onto slides coated with 0.01% Poly-L-Lysine  
325 using a Cytospin cytocentrifuge. Samples were blocked and permeabilized in 2% Triton X-  
326 100 (Sigma-Aldrich) in 5% FBS diluted in PBS. Primary antibodies were applied at room  
327 temperature in 0.25% Triton X-100 in 5% FBS diluted in PBS for 1 hour and then rinsed 3  
328 times with PBS. Secondary antibodies were applied in the same manner. Nuclei were  
329 counterstained with 10 nM DAPI dilactate diluted in PBS for 30 min, samples were rinsed 6  
330 times with PBS, and then mounted in Prolong-Gold with added DAPI (Invitrogen) and  
331 analyzed using a Leica SP8 DLS (Digital light sheet) microscope.

332

333 **TLR/RIG-I stimulations**

334 iPS-DCs were plated at  $1 \times 10^4$  cells per well in 200ul of X-VIVO-15 media without  
335 cytokines. TLR ligands were added directly to the media, and supernatants were harvested  
336 after 24-hour incubation at 37°C. For assays TLR ligands were used at the following  
337 concentrations: Pam3CSK4 (300 ng/mL; InvivoGen), Poly I:C (50 µg/mL; InvivoGen),  
338 Lipopolysaccharide (500 ng/mL; Sigma-Aldrich), Imiquimod (50 µg/mL; InvivoGen); ODN  
339 2216 (3 µg/mL; Miltenyi Biotech). For RIG-I stimulation 1 µg of 3p-hpRNA was complexed  
340 with LyoVec (InvivoGen) for 15 minutes at room temperature and then added to iPS-DCs at  
341 10 ng/mL.

342

343 **Cytokine and chemokine analysis**

344 Human IL-6 and TNF-α protein was measured by ELISA (BioLegend). ELISAs for human  
345 IFN-α and IFN-β were performed on supernatants harvested from mock infected and IAV  
346 infected iPS-DCs using the Verikine Human Interferon Alpha/Interferon Beta ELISA kit  
347 (PBL Assay Science). Murine BAL cytokines were detected using the LEGENDPlex mouse

inflammation panel (13-plex; BioLegend) as per the manufacturer's instructions at 2, 4 and 6 days p.i. and analyzed using the LEGENDPlex analysis software. ELISAs for mouse IFN- $\alpha$  and IFN- $\beta$  were performed on BAL from naïve mice and mice 2 days p.i using VeriKine Mouse Interferon Alpha/Interferon Beta ELISA Kits (PBL Assay Science).

### **Blocking assays**

For blocking assays cells were either pre-incubated for 1 hour with inhibitor (IMD 0354; IKK $\beta$  inhibitor, (Santa Cruz Biotechnology) 5  $\mu$ g/mL) or inhibitor was added directly with viral inoculum (TLR7 inhibitor, ODN 20958, Miltenyi Biotech, 5  $\mu$ M). For type I IFN blocking, cells were pre-incubated with 5  $\mu$ g/mL anti-IFNAR1 antibody (Sigma-Aldrich) for 1 hour prior to infection with A/X-31 influenza without removal of antibody.

### **Statistical analysis**

Statistical significance was performed with GraphPad Prism software. Mann Whitney-U or Student's *t*-tests were used for two-group comparisons. For comparison of IRF5 expression between lung cell subsets identified via CyTOF a repeated measurement one-way ANOVA was used. A *p* value of  $\leq 0.05$  was considered to be significant. For all tests performed, *p* values are reported as n.s.  $> 0.05$ ; \*  $\leq 0.05$ ; \*\*  $\leq 0.01$ ; \*\*\*  $\leq 0.001$ ; \*\*\*\*  $\leq 0.0001$

### **Ethics statement**

All animal studies were performed at Cardiff University (Heath Park research support facility) under UK Home Office Project License number (P7867DADD), as approved by the UK Home Office, London, United Kingdom. Written consent was obtained for the use of cell lines for the HIPSCI project from healthy volunteers. A favorable ethical opinion was granted by the National Research Ethics Service (NRES) Research Ethics Committee Yorkshire and

373 The Humber – Leeds West, reference number 15/YH/0391. Lung tissue samples were  
374 obtained from lung cancer and fibrosis patients from Oxford Radcliffe Biobank with written  
375 consent: a favorable ethical opinion was granted by the South Central-Oxford C Research  
376 Ethics Committee for collection and frozen storage of both tumor and para-tumor lung  
377 samples (Reference number: 09/H0606/5+5).

378

## 379 **Results**

380

### 381 **IRF5 mediates inflammatory cytokine and myeloid cell responses to Influenza A virus** 382 **infection in mice**

383 The mouse is the primary experimental model for studying immunological response to IAV,  
384 where it has been demonstrated that excessive inflammatory cytokine and cellular immune  
385 responses promote lung pathology (2, 26, 27). We first used this model to assess whether  
386 IRF5 impacts influenza-induced immune responses during IAV infection *in vivo*, using the  
387 low pathogenicity murine-adapted H3N2 Influenza A virus (A/X-31). Prior studies have  
388 indicated that viral infections of *Irf5*<sup>-/-</sup> mice lead to reduced cytokine production in  
389 comparison to wild type (WT) controls (14, 17, 28). In accordance, we observed a significant  
390 reduction in early cytokine release in *Irf5*<sup>-/-</sup> mice, with IL-23, IFN- $\gamma$ , TNF- $\alpha$ , MCP-1, IL-6,  
391 IL-17A, IL-1 $\alpha$ , IL-12p70, GM-CSF, IL-1 $\beta$  and IL-27 all significantly reduced in the  
392 Bronchoalveolar Lavage (BAL) of *Irf5*<sup>-/-</sup> mice in comparison to WT controls 2 days post-  
393 infection (p.i) (Fig. 1A), with some cytokines remaining significantly reduced in *Irf5*<sup>-/-</sup> mice 4  
394 days p.i (Fig. 1A). In contrast to other viral infections (17), IFN- $\alpha$  or IFN- $\beta$  production in  
395 response to influenza infection were unaltered (Fig. 1B) at a time-point (day 2 p.i) previously  
396 demonstrated to represent the time of significant A/X-31 influenza-induced type 1 IFN  
397 secretion in this model (29). These data therefore imply that IRF5 selectively modulates



398 expression of certain influenza-induced inflammatory cytokines independently of type I IFNs  
399 in mice.

400

401 Early reduction in inflammatory cytokine production in *Irf5*<sup>-/-</sup> mice was accompanied by a  
402 moderate amelioration of IAV-induced weight loss (Fig. 2A). Interestingly, a recent study  
403 reported that reduced IAV-induced cytokine production in *Irf5*<sup>-/-</sup> mice was associated with  
404 reduced virus replication (28). However, at a time-point where we observed substantially  
405 reduced cytokine production (day 2 p.i), we observed no alteration in IAV load in *Irf5*<sup>-/-</sup> mice  
406 (Fig. 2B), nor did we observe altered virus load in *Irf5*<sup>-/-</sup> mice at a later timepoint of 4 days p.i  
407 (Fig. 2B). Thus, our data demonstrates for the first time that IRF5 promotes IAV-induced  
408 weight loss independently of an impact on influenza replication.

409

410 Given the established role for myeloid cells in pulmonary inflammation during IAV infection  
411 (30, 31), we used polychromatic flow cytometry panels to assess whether *Irf5* influenced  
412 myeloid cell accumulation during infection. Reductions in monocyte-derived DCs, interstitial  
413 macrophages, inflammatory monocytes and conventional DCs in the lungs of *Irf5*<sup>-/-</sup> mice  
414 were observed at 2 days p.i (Fig. 2C). Importantly, lower cytokine responses in *Irf5*<sup>-/-</sup> mice  
415 were accompanied by significant reductions in IL-6<sup>+</sup> cDCs and TNF- $\alpha$ <sup>+</sup> cDCs, interstitial  
416 macrophages, cDCs and pDCs in the airways (Fig. 2D). Thus, these data suggested that *Irf5*  
417 plays a key role in shaping the early innate inflammatory response during influenza infection  
418 and point to a central role for myeloid cells in promoting IRF5-driven viral disease.

419

#### 420 **Myeloid cells in human lung express high levels of IRF5**

421 Although the mouse is a useful model for probing immune responses to IAV, numerous  
422 differences exist between the mouse and human immune system. It was therefore important

423 to investigate the role of human IRF5 in IAV-induced immune responses. We first measured  
424 IRF5 expression in multiple cell subsets in human lung samples using CyTOF (Fig. 3A).  
425 Using lung samples from four independent donors, we identified significantly different IRF5  
426 expression dependent on cell subset ( $p = 0.0252$ , R-Square = 0.9098) (Fig. 3B), with cells of  
427 the myeloid lineage, particularly eosinophils, basophils and monocytes, displaying highest  
428 expression of IRF5 in human lung. Expression was higher in CD1c<sup>+</sup> DCs and CD141<sup>+</sup> DCs  
429 than in lung resident macrophages, where expression was relatively low. Furthermore, when  
430 IRF5 expression data was combined for all myeloid cell subsets and all lymphoid cell subsets  
431 (Fig. 3C), expression of IRF5 was significantly higher in myeloid cells in comparison to  
432 lymphoid cells (median expression myeloid = 6.01, median expression lymphoid = 1.79;  $p =$   
433  $<0.0001$ ), suggesting that IRF5 expression is highest in the myeloid compartment.

434

435 **iPSCs with a biallelic mutation in *IRF5* can be differentiated into conventional dendritic**  
436 **cells**

437 Given that, in the human lung, IRF5 expression was highest in cells of the myeloid lineage  
438 and that in mice *Irf5* promoted pro-inflammatory cytokine production by myeloid cells in  
439 response to IAV infection, we next sought to establish a human myeloid cell model to  
440 scrutinize the role of IRF5 in myeloid cell cytokine response to IAV. We differentiated a  
441 hPSC line with a biallelic mutation in *IRF5* generated using CRISPR/Cas9 genome editing  
442 (16) and the parental line Kolf2, into iPS-DCs using a published differentiation strategy (24).  
443 We also generated a complemented isogenic control line for *IRF5*<sup>-/-</sup> (hereafter “*IRF5*<sup>Comp</sup>”) to  
444 confirm IRF5-dependency of any phenotypes observed (32). To confirm gene editing  
445 strategies, we examined the expression of IRF5 in Kolf2, *IRF5*<sup>-/-</sup> and *IRF5*<sup>Comp</sup> iPS-DCs.  
446 *IRF5* mRNA was detected in Kolf2 iPS-DCs but not *IRF5*<sup>-/-</sup> iPS-DCs, with expression  
447 restored in *IRF5*<sup>Comp</sup> iPS-DCs (Fig. 4A). Similar restoration of IRF5 protein expression in

448 IRF5<sup>Comp</sup> iPS-DCs was observed (Fig. 4B). Furthermore, after infection of iPS-DCs with  
449 IAV, IRF5 was detected in Kolf2 iPS-DCs and not IRF5<sup>-/-</sup> iPS-DCs by immunostaining (Fig.  
450 4C). We then compared IAV-induced cytokine production by iPS-DCs from our healthy  
451 control iPSC line Kolf2 with monocyte-derived DCs from the blood of three healthy donors  
452 which were left either immature or matured for 48 hours with LPS. iPS-DCs demonstrated  
453 similar cytokine profiles after IAV infection to immature monocyte-derived DCs (Fig. 4D),  
454 validating iPS-DCs as an experimental system for examining virus-induced cytokine  
455 responses.

456

457 It has previously been shown that IRF5 deficiency or TALEN-based engineering targeting  
458 the AAVS1 viral integration site does not affect the differentiation capacity of iPSCs to iPS-  
459 derived macrophages (iPSDMs) (16, 33). To ensure that genome editing strategies had not  
460 altered the differentiation capacity of iPSCs to dendritic cells, we assessed the differentiation  
461 efficiency of Kolf2, IRF5<sup>-/-</sup> and IRF5<sup>Comp</sup> iPSCs. We observed similar numbers of cells  
462 harvested from embryoid bodies (EBs) from day 19-24 of differentiation, with no significant  
463 difference in the number of cells harvested from eight independent differentiations per line  
464 (Fig. 5A). After completion of the 25-day DC differentiation, DC marker expression was  
465 examined by flow cytometry, with CD141, CLEC9A, CD11c, MHC II and CD86 similarly  
466 expressed in all three iPS-DC lines (Fig. 5B). There are three main subsets of human DCs,  
467 pDCs and two subsets of myeloid (conventional) DCs, CD1c<sup>+</sup> and CD141<sup>+</sup>, with DC  
468 hematopoiesis distinct from the development of monocytes (34). iPS-DCs express markers  
469 of human conventional DCs including CD11c and CD141 (Fig. 5B), as well as HLA-DR,  
470 CD86 and CLEC9A, which have been shown to be expressed by human CD141<sup>+</sup> DCs (35).  
471 We did not detect CD303 expression, a marker for pDCs or CD1c, the marker for the other  
472 subset of human conventional dendritic cells (36). As observed by Sachamitr et al (24) we

also detected CD14 and DC-SIGN expression by iPS-DCs (Forbester J & Humphreys I, unpublished data). Gene expression analysis confirmed similar induction of the DC markers *CD83* and *CD86* in all three iPSC lines after differentiation to iPS-DCs, and loss of expression of the pluripotency markers *NANOG* and *POU5F1* (Fig. 5C). Morphology of Klf2, IRF5<sup>-/-</sup> and IRF5<sup>Comp</sup> iPS-DCs in culture was indistinguishable (Fig. 5D). Taken together, these data suggest that neither IRF5 deficiency nor TALEN-based engineering influence iPSC differentiation.

#### **IRF5 enhances IAV-induced inflammatory cytokine production in iPS-DCs**

After confirming that IRF5 deficiency did not alter iPS-DC surface phenotype or morphology (Fig. 5) and that iPS-DCs exhibit similar cytokine profiles to human monocyte-derived DCs after IAV stimulation (Fig. 4D), we next used iPS-DCs to determine whether IRF5 has a cell-intrinsic role in human DC cytokine production, in particular the pro-inflammatory cytokines IL-6 and TNF- $\alpha$ . Despite a protective role for IL-6 being reported in murine models of IAV infection (37, 38), high production of IL-6 is linked to severity of symptoms in humans patient cohorts (39, 40), whereas TNF- $\alpha$  has been shown to enhance cellular inflammation and pathology during IAV infection (26).

24 hours after stimulation of iPS-DCs with IAV, IL-6 and TNF- $\alpha$  production by IRF5<sup>-/-</sup> iPS-DCs was significantly reduced in comparison to Klf2 iPS-DCs whereas cytokine production was restored in IRF5<sup>Comp</sup> iPS-DCs (Fig. 6A, iPS-DCs). IRF5 deficiency had no impact on virus entry, as indicated by comparable influenza nucleoprotein (NP) staining after 24 hours (Fig. 6B & C). In addition, there was no significant difference in cell viability after IAV stimulation, as measured by live viability dye and flow cytometry 24h p.i. (Mean  $\pm$  SEM % live cells: IRF5<sup>Comp</sup>, 65.767  $\pm$  0.353; IRF5<sup>-/-</sup>, 63.95  $\pm$  1.655; Klf2, 70  $\pm$  1.654). Surface

analysis of DC markers by flow cytometry showed that the number of iPS-DCs expressing DC maturation markers after IAV infection was similarly significantly increased in Kolf2, IRF5<sup>-/-</sup> and IRF5<sup>Comp</sup> iPS-DCs (Fig. 6D). Moreover, gene complementation has previously been used to inhibit immune responsiveness in the context of restoration of expression of the inhibitory IL-10 receptor into *IL10RB*<sup>-/-</sup> iPS-derived myeloid cells (33). Thus we do not believe that restored cytokine responsiveness of complemented IRF5<sup>-/-</sup> cells is a consequence of non-specific induction of cytokine secretion by the complementation process, but instead is due to IRF5 itself. Collectively, these data suggest that IRF5 deficiency selectively alters iPS-DC cytokine production after exposure to IAV. In addition, to probe IRF5 deficiency in a different myeloid cell lineage, we differentiated IRF5<sup>-/-</sup>, Kolf2 and IRF5<sup>Comp</sup> iPSCs to macrophages (iPSDMs) using a slightly modified version of a previously published protocol (22), demonstrating a similar significant reduction in IL-6 and TNF- $\alpha$  production as observed in iPS-DCs (Fig. 6A, iPSDMs).

511

### **IRF5 acts downstream of TLR7 and, possibly, RIG-I signalling pathways to drive human myeloid cell cytokine responses to IAV**

In some experimental systems, IRF5 mediates virus-induced production of type I IFN (17, 41). Given that type I IFN is implicated in driving influenza-induced inflammatory cytokine responses (42), we assessed whether IRF5 deficiency impacted influenza-induced IFN production. Blocking type I IFN reduced IAV-induced IL-6 and TNF- $\alpha$  production albeit, in the case of IL-6, not to levels produced by IRF5<sup>-/-</sup> iPS-DCs (Fig. 7A). Furthermore, IRF5 had no impact on type I IFN secretion by iPS-DCs (Fig. 7B). Thus, although type I IFN enhanced IRF5-induced pro-inflammatory cytokine secretion, the production of type I IFN by iPS-DCs was not an IRF5-regulated process.

522

523 We next investigated which PRRs require IRF5 to elicit cytokine responses following IAV  
524 stimulation of iPS-DCs. IAV is detected by endosomal TLR7, and members of the DExDC  
525 helicase family and RIG-I in dendritic cells (43–45). Consistent with data from human and  
526 murine macrophages (12, 46), IRF5<sup>-/-</sup> iPS-DCs produced significantly less IL-6 in response to  
527 agonists of TLR7 (and TLRs 4, 3 and 9, Fig. 8A). Moreover, stimulation of IRF5<sup>-/-</sup> iPS-DCs  
528 with the RIG-I-specific agonist 3p-hpRNA led to a significant reduction in IL-6 production  
529 (Fig. 8B), demonstrating that IRF5 mediates RIG-I- and TLR7-induced responses in iPS-  
530 DCs. Substantial IFN-dependent induction of the RIG-I encoding gene *DDX58* and, to a  
531 lesser extent, *TLR7* were observed upon IAV stimulation of iPS-DCs (Fig. 8C & D), and  
532 IRF5 deficiency did not impair baseline PRR expression (Fig. 8E), suggesting that reduced  
533 expression of pro-inflammatory cytokines in response to TLR7, RIG-I and IAV stimulation  
534 in IRF5<sup>-/-</sup> iPS-DCs was not a consequence of restricted PRR expression by these cells.

535

536 Finally, we wanted to determine which PRRs mediated IAV-induced cytokine responses in  
537 iPS-DCs. Because 1) there is no selective antagonist of RIG-I and 2) human CD141<sup>+</sup> DCs  
538 express TLR7 (47), we focused on the role of TLR7 in mediating IAV-induced iPS-DC  
539 cytokine responses. Addition of the specific TLR7 antagonist ODN 20958 to IAV-stimulated  
540 Kolf2 iPS-DCs significantly abated IL-6 production whereas TLR7 inhibition in IRF5<sup>-/-</sup> iPS-  
541 DCs did not further reduce IAV-induced IL-6 responses (Fig. 8F). These data suggest that  
542 IRF5 promotes TLR7 mediated cytokine responses following IAV detection by human DCs.  
543 However, IAV-induced cytokine secretion was incompletely inhibited by TLR7 blockade in  
544 Kolf2 iPS-DCs, suggesting that other PRRs contribute to IRF5-mediated responses. IKK $\beta$   
545 has been shown to play a crucial role in IRF5 and NF- $\kappa$ B activation (11). In support of this,  
546 pre-treatment of IAV-stimulated iPS-DCs with the IKK $\beta$  inhibitor IMD 0354 dramatically  
547 reduced IAV-induced cytokine production by iPS-DCs (Fig. 8B) implying that other PRRs

548 including, possibly, RIG-I contribute to IAV-induced cytokine responses by iPS-DCs in  
549 addition to TLR7.

550

## 551 Discussion

552 Here, using iPS-DCs as a model system, we have shown that IRF5 expression by myeloid  
553 cells is important in driving the inflammatory response to IAV, without impacting viral  
554 uptake by iPS-DCs or DC maturation. Using various blocking assays and stimulation with  
555 TLR/RLR ligands, we show that IRF5 is most likely acting downstream of TLR7 and,  
556 possibly, RIG-I signaling to drive the production of pro-inflammatory cytokines.

557

558 Given that IFN-stimulated genes contribute to anti-influenza immunity (48), to identify  
559 whether IRF5 and/or related pathways could be safely exploited to dampen inflammatory  
560 cytokine responses to influenza it is important to understand the relationship between IRF5  
561 and virus-induced type 1 IFN. We found that IRF5<sup>-/-</sup> iPS-DCs and *Irf5*<sup>-/-</sup> mice were not  
562 deficient in type I IFN production, but that type I IFN enhances the IRF5-mediated  
563 inflammatory cytokine response, a process associated with IFN-mediated induction of TLR7  
564 and RIG-I. Although certain studies have reported a role for IRF5 in type I IFN induction  
565 directly in certain contexts (28, 46, 49) functional redundancy between IRF proteins may  
566 exist [46]. Also, although a role for IRF5 in promoting type 1 IFN secretion following  
567 influenza infection *in vivo* has been reported, the same studies observed reduced virus  
568 replication in *Irf5*<sup>-/-</sup> mice, precluding the possibility to uncouple decreased virus replication  
569 and subsequent pattern recognition receptor stimulation from a direct influence of IRF5 on  
570 type 1 IFN expression. Furthermore, early *in vivo* studies of *Irf5* responses to viruses may  
571 have been confounded by a *Dock2* mutation prevalent in *Irf5*<sup>-/-</sup> mouse colonies (50). Also, it  
572 has been demonstrated *in vitro* that, unlike IRF3 and IRF7, IRF5 does not bind to the virus-

573 response elements in IFN promoters (51). IRF5-mediated induction of type I IFN may also be  
574 virus-specific, at least *in vitro*, with NDV, VSV and HSV-1 infection shown to activate IRF5  
575 but lead to induction of distinct IFNA gene subtypes in human cell lines (52). IRF3 and IRF7  
576 are activated by IAV, and these transcription factors have been shown to be necessary for  
577 inducing type I IFN after IAV infection (53, 54). Therefore, we suggest that after sensing of  
578 IAV by dendritic cells, IRF3, IRF5, and IRF7 are induced, resulting in the production of type  
579 I IFN and inflammatory cytokines, with the type I IFN induced by IRF3 and IRF7  
580 exacerbating IRF5 activation, likely in part through induction of TLR7 and, possibly, RIG-I  
581 expression. In the context of influenza pathogenesis, our data imply that IRF5 could be safely  
582 targeted to limit virus-induced pro-inflammatory cytokine production without affecting IFN  
583 production and associated induction of antiviral effector genes.

584

585 The phenotype of reduced inflammatory cytokines observed in our iPS-DC model was also  
586 evident *in vivo* using *Irf5*<sup>-/-</sup> mice. Although we observed no mortality in either WT or *Irf5*<sup>-/-</sup>  
587 mice (Forbester J & Humphreys I, unpublished data), we observed reduced cytokine  
588 production by myeloid cells that correlated with reduced cellular pulmonary infiltration and a  
589 moderate impact on virus-induced weight loss. Although *Irf5*<sup>-/-</sup> mice have previously been  
590 shown to be less susceptible to IAV-induced pathology (28), in this study we were able to de-  
591 couple viral load and inflammatory cytokines in the early stages of infection, demonstrating  
592 that the enhanced pathology in WT compared to *Irf5*<sup>-/-</sup> mice was immune-mediated rather  
593 than a consequence of heightened virus replication. Why differences exist between our data  
594 and those obtained by Chen *et al* (28) is unclear, although this may reflect the different  
595 influenza strains (H3N2 versus H1N1) used in these experiments. Irrespective, the data  
596 presented herein demonstrates that IRF5 modulates viral pathogenesis via the regulation of  
597 inflammation and not virus replication, and that targeting this pathway as an adjunct therapy



598 to antiviral drug treatment may represent an effective therapeutic approach to treatment of  
599 influenza pathogenesis.

600

601 Although the mouse provides a useful model to study viral pathogenesis there are inherent  
602 immunological differences between mice and humans (55). Therefore, we wanted to establish  
603 a human cell system amenable to genetic manipulation, so that gene function can be  
604 understood in the context of a human cellular environment. Given that primary human  
605 myeloid cells are difficult to genetically manipulate and access in large numbers, hiPSCs  
606 offer a solution; once hiPSCs are generated they can be differentiated down multiple cellular  
607 lineages, providing the opportunity to study gene function in multiple different cell types,  
608 with a defined genetic background. In addition, as hiPSCs are self-renewing, starting material  
609 is an unlimited resource. Furthermore, many research groups have shown that hiPSCs can be  
610 relatively easily genetically manipulated using tools such as Zinc finger nucleases, TALENs  
611 and CRISPR-Cas9 systems (56). Here, we show that iPSCs can be differentiated into DCs  
612 expressing markers of human CD141<sup>+</sup> myeloid DCs. However, the levels of the specific  
613 lineage markers for CD141<sup>+</sup> DCs, CLEC9A and XCR1 were quite low in our DC  
614 populations, which has been previously described (24). The complex environment and array  
615 of signals DC progenitors are exposed to during development presents a challenge to  
616 replicate *in vitro*. However, fundamental understanding of human DC development is  
617 expanding, and in the future knowledge of detailed changes in the transcriptional profile of  
618 these cells during development can be applied to help refine differentiation protocols.  
619 However, iPS-DCs described herein expressed multiple DC lineage markers, suggesting that  
620 our differentiation protocol is sufficient to derive DC-like cells. In addition, we demonstrated  
621 that after differentiation into iPSDMs, IRF5<sup>-/-</sup> cells are also deficient in IL-6 and TNF- $\alpha$   
622 production, demonstrating for the first time that virus induced immune responses, including

623 cytokine secretion, can be investigated in iPS-derived cells of multiple myeloid lineages that  
624 contain biallelic mutations, thus demonstrating the flexibility of iPSCs as tools to study  
625 immune responses to pathogens in multiple cell types.

626

627 In two independent studies response eQTLs (reQTLs) were found in IRF5 after stimulation of  
628 cells with virus or TLR ligands (57, 58), suggesting that variation within the IRF5 locus may  
629 be important in driving differences in expression. It would be interesting in future studies to  
630 see if SNPs which drive higher IRF5 expression in human macrophages and DCs also  
631 correlate with a heightened inflammatory response to viruses such as IAV, as our data  
632 suggest that such individuals may be preferentially susceptible to influenza pathogenesis and  
633 imply that targeting high IRF5 levels in these individuals could reduce inflammation without  
634 impacting virus control. As well as being a useful tool to knockout genes to assess cell-  
635 specific function as we have shown here, iPS-DCs could also be used as a tool to explore  
636 how common human genetic variants are associated with immune cell responses to various  
637 pathogens.

638

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652

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654 S.C., C.H. performed experiments using human iPSCs. A.Y. generated TALEN-engineered  
655 IRF5<sup>-/-</sup> iPSCs. J.L.F. and D.W. performed experiments using human blood-derived DCs.  
656 D.W. conducted CyTOF experiments and analyzed CyTOF data. I.U. provided *Irf5*<sup>-/-</sup> mice.  
657 J.L.F., I.R.H., I.U., M.C., D.W. designed experiments. I.R.H., T.D. & G.D. supervised the  
658 study. J.L.F. and I.R.H wrote the manuscript. All authors approved the final manuscript.

659

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860 **Figure legends**

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862 **Figure 1. IRF5 alters cytokine responses to Influenza A virus in a murine infection**

863 **model.** WT and *Irf5*<sup>-/-</sup> mice were infected intranasally with 3 x 10<sup>3</sup> A/X-31 influenza (A)  
864 Inflammatory cytokine expression in BAL, was measured using multiplex assays 2, 4 and 7  
865 days p.i. Data shown as mean ± SEM using 7 WT and 5 *Irf5*<sup>-/-</sup> mice (day 2) or five mice per  
866 genotype (day 4 and day 7) and represents two independent experiments. (B) IFN-α and IFN-  
867 β levels in BAL measured by ELISA in *Irf5*<sup>-/-</sup> and WT naïve and IAV infected mice 2 days  
868 p.i. Data shown as mean ± SEM of 3-6 mice per group at 2 days p.i.

869

870 **Figure 2. IRF5 enhances Influenza A virus-induced inflammatory response in a murine**

871 **infection model.** (A) Weight loss of WT and *Irf5*<sup>-/-</sup> mice was assessed over time and  
872 comparable results were observed in 4 independent experiments, with 4-5 WT or *Irf5*<sup>-/-</sup> mice  
873 in each group per experiment. Data shown as mean ± SEM. (B) Replicating virus in lung was  
874 quantified by plaque assay. Data shown as mean ± SEM using 7 WT and 5 *Irf5*<sup>-/-</sup> mice for day  
875 2, and 5 mice of each genotype for day 4. (C) Recruitment of specific myeloid cell  
876 populations (mDCs, monocyte-derived DCs; cDCs, conventional DCs; pDCs, plasmacytoid  
877 DCs; Inflam. mon, inflammatory monocytes) in WT and *Irf5*<sup>-/-</sup> mice was assessed by flow  
878 cytometry 2 days p.i. Populations were defined by the following markers: Alveolar  
879 macrophages - SiglecF<sup>+</sup> CD11b<sup>+</sup> CD64<sup>+</sup> Ly6C<sup>-</sup>; mDCs - SiglecF<sup>-</sup> CD11b<sup>+</sup> MHC II<sup>+</sup> CD11c<sup>+</sup>  
880 CD64<sup>+</sup> Ly6C<sup>+</sup>; Interstitial macrophages - SiglecF<sup>-</sup> CD11b<sup>+</sup> MHC II<sup>+</sup> CD11c<sup>-</sup> CD64<sup>+</sup> Ly6C<sup>+</sup>;  
881 Inflammatory monocytes - Siglec F<sup>-</sup> CD11b<sup>+</sup> MHC II<sup>-</sup> Ly6C<sup>+</sup> CD64<sup>+</sup>; cDCs; MHC II<sup>+</sup>  
882 CD11c<sup>+</sup> Ly6C<sup>-</sup>; pDCs – B220<sup>+</sup> SiglecH<sup>+</sup> MHC II<sup>low</sup> CD11c<sup>low</sup>; Eosinophils – SiglecF<sup>+</sup>  
883 CD11c<sup>-</sup> CD11b<sup>+</sup> Ly6C<sup>-</sup>. Data shown as mean ± SEM using 11 WT and 10 *Irf5*<sup>-/-</sup> mice from  
884 multiple replicates. (D) The total number of each individual myeloid cell population

(unstimulated, ex vivo) positive for IL-6 and TNF- $\alpha$  expression by was detected by flow cytometry, with data presented representing mean total cell number per  $10^5$  cells of each cell type  $\pm$  SEM. Data represents two experiments.

**Figure 3. IRF5 expression in human lung cells.** IRF5 expression by multiple cellular subsets derived from human lung tissue from independent donors was analyzed by CyTOF. (A) UMAP based on down sampled, concatenated files from lung samples from four donors using phenotypic markers. Post UMAP analysis, populations (colored by cell type as identified by lung CyTOF) were defined via the following markers: CD4<sup>+</sup> T cells, CD3<sup>+</sup> CD4<sup>+</sup> CD20<sup>-</sup>; CD8<sup>+</sup> T cells, CD3<sup>+</sup> CD20<sup>-</sup> CD8<sup>+</sup>; B cells, CD3<sup>-</sup> CD20<sup>+</sup>; NK cells, CD3<sup>-</sup> CD20<sup>-</sup> CD56<sup>+</sup>; CD14<sup>+</sup> Monocytes, CD16<sup>-</sup> CD11b<sup>+</sup> CD14<sup>+</sup> HLA-DR<sup>+</sup>; CD16<sup>+</sup> Monocytes, CD14<sup>-</sup> CD11b<sup>+</sup> CD16<sup>+</sup> HLA-DR<sup>+</sup>; Macrophages, CD11b<sup>+</sup> CD68<sup>+</sup> HLA-DR<sup>+</sup>; pDCs, CD123<sup>+</sup> CD11b<sup>+</sup> HLA-DR<sup>+</sup>; CD141<sup>+</sup> cDCs, CD11b<sup>+</sup> HLA-DR<sup>+</sup> CD1c<sup>-</sup> CD141<sup>+</sup>; CD1c<sup>+</sup> cDCs, CD11b<sup>+</sup> HLA-DR<sup>+</sup> CD1c<sup>+</sup> CD141<sup>-</sup>; Eosinophils, Siglec8<sup>+</sup> CD123<sup>-</sup>; Basophils, Siglec8<sup>+</sup> CD123<sup>+</sup> (B) Median IRF5 expression in populations identified in (A) from lung samples taken from four independent donors, corrected for non-specific staining using unpermeabilized controls for each sample, error bars represent  $\pm$  SEM. (C) Median IRF5 expression in myeloid vs lymphoid cell subsets, error bars represent  $\pm$  SEM.

**Figure 4. *IRF5*<sup>-/-</sup> iPSCs, *IRF5*<sup>Comp</sup> iPSCs and Kolf2 iPSCs can be differentiated into iPS-DCs which lack or express IRF5.** CRISPR/Cas9 was used to generate biallelic mutations in *IRF5* in the Kolf2 background. *IRF5*<sup>Comp</sup> iPSCs were generated using TALEN-mediated integration of *IRF5* into the *IRF5*<sup>-/-</sup> background. (A) Relative expression of *IRF5* in iPSCs and iPS-DCs, relative to *GAPDH*. Data shown as four technical replicates per assay, with assays repeated three times from independent iPS-DC batches. (B) Flow cytometry showing

910 IRF5 expression in iPS-DCs generated from IRF5<sup>-/-</sup>, IRF5<sup>Comp</sup> and Kolf2 iPSCs. (C)  
911 Immunostaining for IRF5 in A/X-31 influenza (IAV) infected Kolf2 and IRF5<sup>-/-</sup> iPS-DCs  
912 (DAPI, blue; IRF5, green). (D) IL-6 and TNF- $\alpha$  production 24h p.i. by A/X-31 influenza  
913 (IAV) challenged Kolf2 iPS-DCs and monocyte-derived DCs generated from human  
914 peripheral blood, either with or without 48h LPS maturation, was assayed by ELISA. Data  
915 represented shows mean  $\pm$  SEM from three independent Kolf2 differentiations for iPS-DCs,  
916 and from three independent healthy donors for monocyte-derived DCs.

917

918 **Figure 5. IRF5<sup>-/-</sup> iPSCs, IRF5<sup>Comp</sup> iPSCs and Kolf2 iPSCs can be differentiated into iPS-**  
919 **DCs that display similar morphology.** IRF5<sup>-/-</sup> iPSCs, IRF5<sup>Comp</sup> and Kolf2 iPSCs were  
920 differentiated into dendritic cells using defined concentrations of growth factors to generate  
921 embryoid bodies (EBs), and GM-CSF and IL-4 to generate immature DCs from these EBs.  
922 (A) Total cell numbers of DC precursors harvested from DC differentiation plates. Data  
923 shown as 8 independent differentiations per iPSC line. (B) Surface expression of DC markers  
924 was examined via flow cytometry in Kolf2, IRF5<sup>-/-</sup> and IRF5<sup>Comp</sup> iPS-DCs. Representative  
925 plots presented from one experiment, with experiments performed at least three times. (C)  
926 Gene expression of DC markers *CD83* and *CD86* and iPSC markers *NANOG* and *POU5F1*  
927 by iPS-DCs, relative to *GAPDH* was quantified using TaqMan gene-expression assays. Data  
928 shown represents four technical replicates per assay, with assays repeated at least twice from  
929 independent iPS-DC batches. (D) Morphology of iPS-DCs generated from Kolf2, IRF5<sup>Comp</sup>  
930 and IRF5<sup>-/-</sup> iPSCs.

931

932 **Figure 6. IRF5 enhances IAV-induced inflammatory cytokine production in iPS-DCs**  
933 **and iPSDMs.** (A) IL-6 and TNF- $\alpha$  were measured by ELISA in supernatants harvested from  
934 iPS-DCs and iPSDMs generated from an iPSC line with a biallelic mutation in *IRF5*,

935 compared to the parent line Kolf2, and a line with a functional *IRF5* gene reintroduced into  
936 the AAVS1 integration site by TALEN engineering, after infection with IAV at MOI 1.  
937 Supernatants were harvested at 24 hours for assays; data shown represents mean  $\pm$  SEM for  
938 triplicate wells from at least 3 independent experiments. **(B)** *IRF5*<sup>-/-</sup>, *IRF5*<sup>Comp</sup> and Kolf2 iPS-  
939 DCs were infected with IAV at MOI 1, and then stained for IAV NP 24 hours post-infection  
940 and analyzed via flow cytometry **(C)** % positive NP iPS-DCs 24-hour IAV post-infection,  
941 with data presented showing mean  $\pm$  SEM from three independent experiments. **(D)**  
942 Expression of DC maturation surface markers for iPS-DCs generated from *IRF5*<sup>-/-</sup>, Kolf2 or  
943 *IRF5*<sup>Comp</sup> hIPSCs 24-hour post-infection with A/X-31 influenza (IAV), MOI 1, as measured  
944 by flow cytometry, with data presented showing mean  $\pm$  SEM from three independent  
945 experiments.

946

947 **Figure 7. Type I IFN signaling enhances IL-6 and TNF- $\alpha$  production by iPS-DCs.** 2 x  
948 10<sup>4</sup> iPS-DCs were challenged as stated below for each assay, and supernatants were  
949 harvested after 24 hours, unless otherwise stated. A/X-31 influenza (IAV) was used at MOI  
950 1. **(A)** Cells were pre-incubated for 1-hour with anti-IFNAR1 antibody, or left untreated prior  
951 to viral infection. Data shown represents mean  $\pm$  SEM for triplicate wells from at least 3  
952 experiments. Supernatants were harvested and assayed for IL-6 and TNF- $\alpha$  by ELISA. **(B)**  
953 Supernatants from mock or IAV-infected Kolf2 or *IRF5*<sup>-/-</sup> iPS-DCs were harvested at 24  
954 hours and assayed for IFN- $\alpha$  and IFN- $\beta$  by ELISA. Data shown represents 2 separate  
955 experiments.

956

957 **Figure 8. IRF5 acts downstream of TLR7 and RIG-I to drive inflammatory cytokine**  
958 **responses in iPS-DCs.** 2 x 10<sup>4</sup> iPS-DCs were challenged as stated below for each condition  
959 in each assay and supernatants were harvested after 24 hours. A/X-31 influenza (IAV) was

960 used at MOI 1. For blocking assays cells were either pre-incubated for 1 hour with inhibitor  
961 (IMD 0354; IKK $\beta$  inhibitor) or inhibitor was added directly with viral inoculum (ODN  
962 20958; TLR7 inhibitor). Data shown represents mean  $\pm$  SEM for triplicate wells from at least  
963 3 experiments, unless otherwise stated. **(A)** IL-6 production by Kolf2 and IRF5<sup>-/-</sup> iPS-DCs in  
964 response to stimulation with various TLR ligands (TLR2: Pam3CSK4, 300 ng/mL; TLR3:  
965 Poly I:C, 50  $\mu$ g/mL; TLR4: LPS, 50  $\mu$ g/mL; TLR7: Imiquimod, 50  $\mu$ g/mL; TLR9: ODN  
966 2216, 3  $\mu$ g/mL) was measured by ELISA. Data shown represents four wells per condition for  
967 one iPS-DC batch per line, with assays replicated in two independent experiments. **(B)** IL-6  
968 response as measured by ELISA in Kolf2 and IRF5<sup>-/-</sup> iPS-DCs to RIG-I ligand 3p-hpRNA  
969 with or without IKK $\beta$  inhibitor IMD 0354; and to A/X-31 influenza (IAV) with or without  
970 IMD 0354. **(C)** Fold-change in mRNA levels for *TLR7* and *DDX58*, measured by RT-qPCR  
971 using *GAPDH* as an endogenous control. **(D)** *DDX58* and *TLR7* mRNA levels in iPS-DCs  
972 after A/X-31 influenza (IAV) infection with or without blocking of type I IFN signaling  
973 using anti-IFNAR1. Data shown represents four technical replicates per assay, with assays  
974 repeated at least twice from independent iPS-DC batches. **(E)** Relative mRNA levels of *TLR7*  
975 in iPS-DCs generated from IRF5<sup>-/-</sup> iPSCs or parent Kolf2 iPSCs, measured using RT-qPCR.  
976 **(F)** IL-6 response as measured by ELISA in Kolf2 and IRF5<sup>-/-</sup> iPS-DCs to A/X-31 influenza  
977 with or without TLR7 inhibitor ODN 20958.  
978



Figures

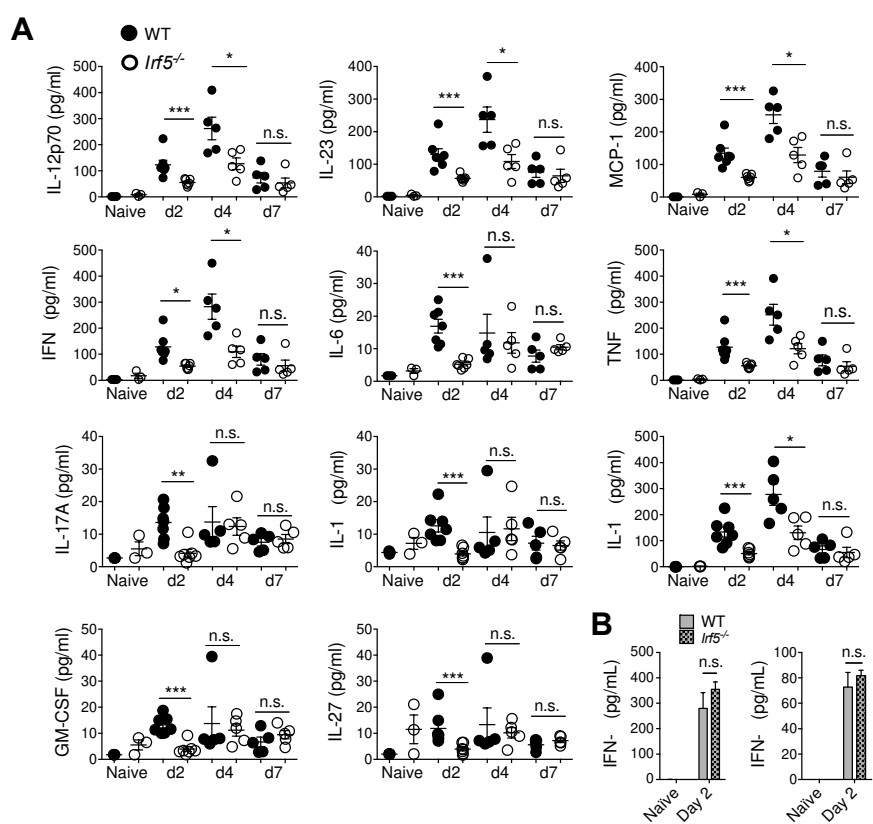


Figure 1.

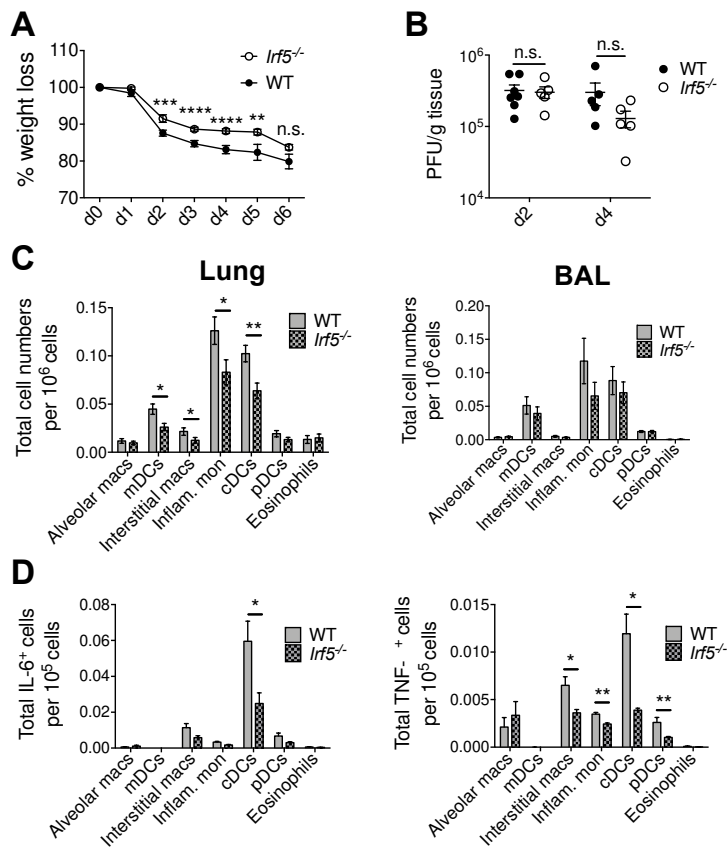
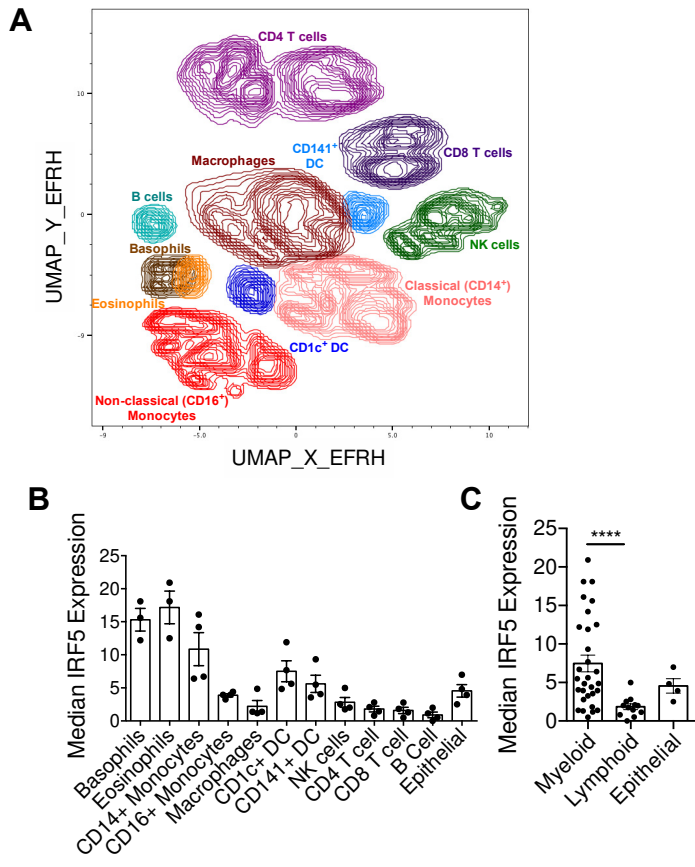


Figure 2.



**Figure 3.**

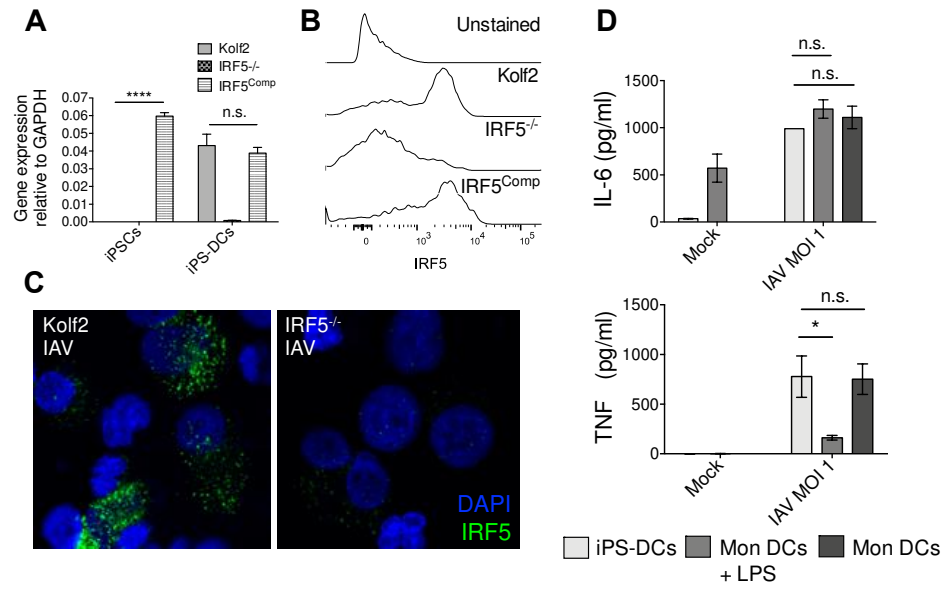


Figure 4.

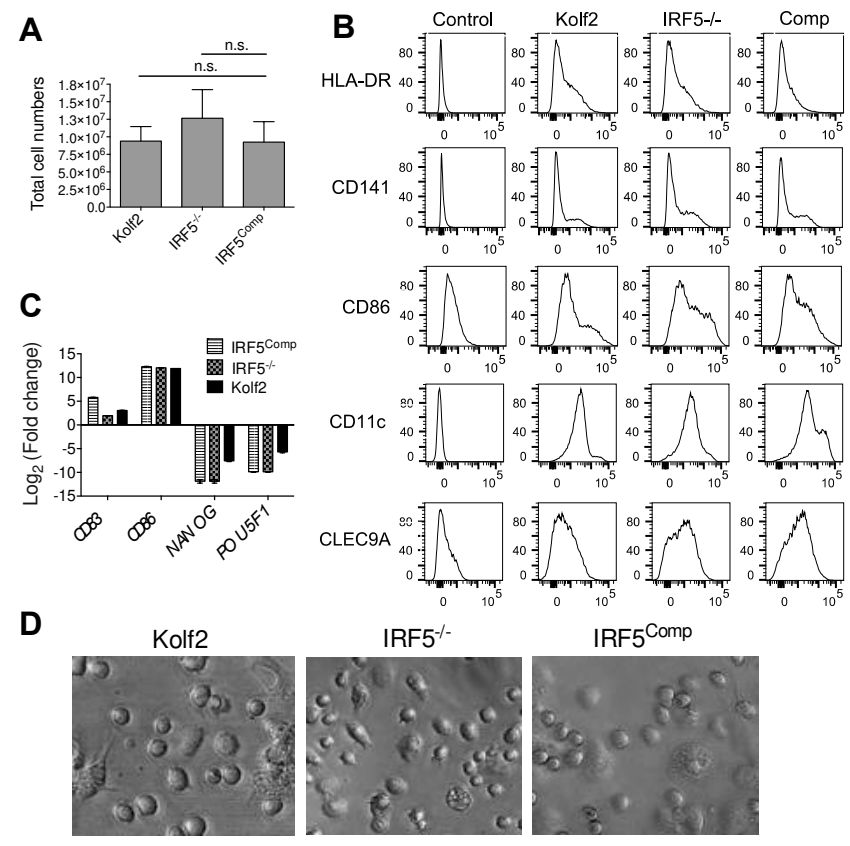


Figure 5.

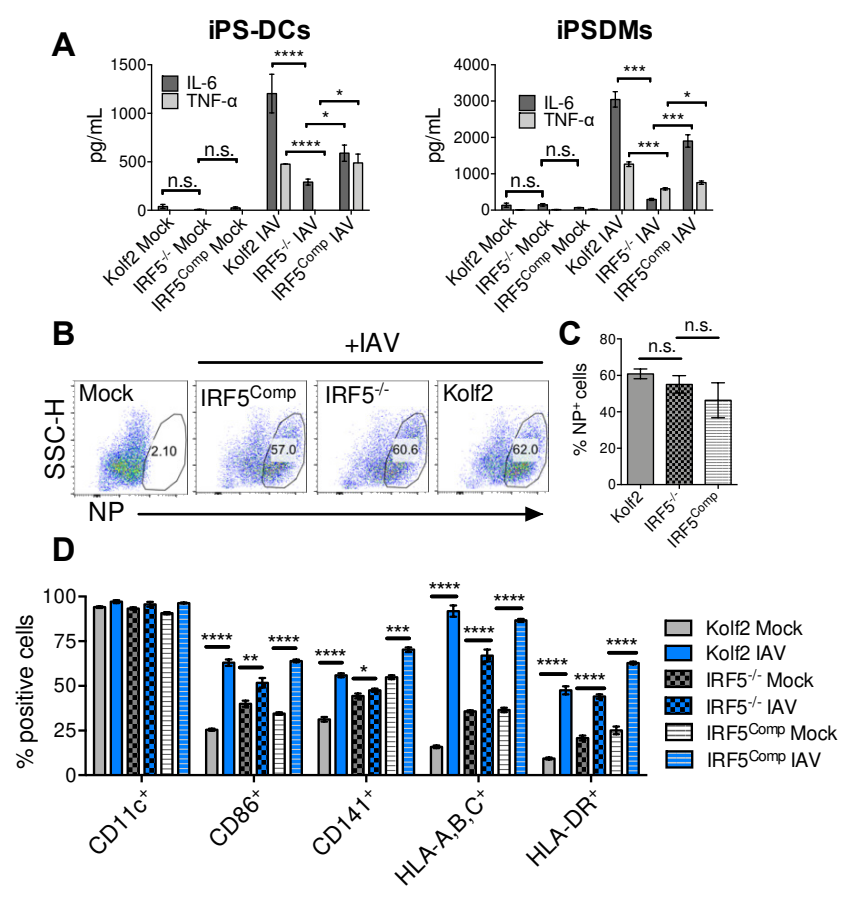


Figure 6.

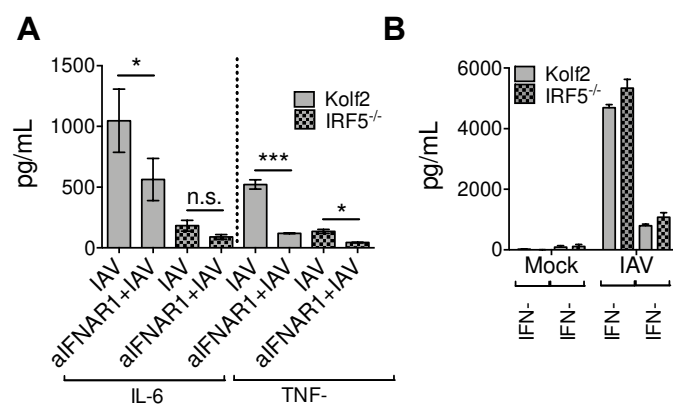


Figure 7.

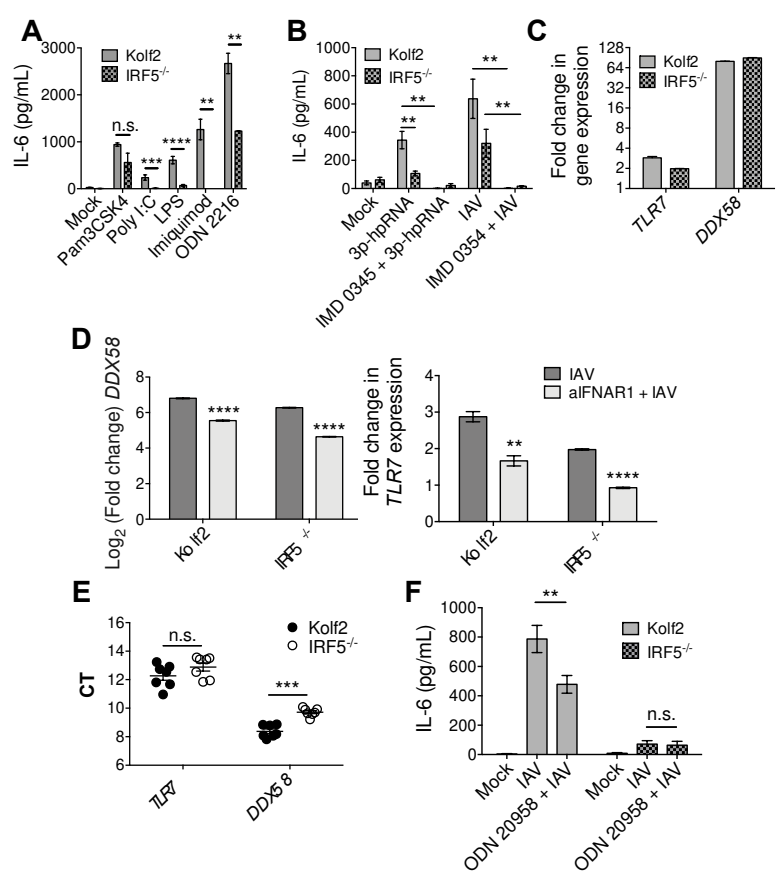


Figure 8.